Vaccine 34 (2016) 1054–1061

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Recombinant lipidated dengue-3 envelope protein domain III stimulates broad immune responses in mice

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ARTICLE INFO

Article history:
Received 12 August 2015
Received in revised form
23 December 2015
Accepted 5 January 2016
Available online 15 January 2016

Keywords:
Dengue virus
Envelope protein domain III
Lipoprotein
Vaccine

ABSTRACT

The linkage of an immunogen with a toll-like receptor ligand has great potential to induce highly potent immune responses with the initial features of antigen-presenting cell activation. In the current study, we expressed recombinant dengue-3 envelope protein domain III (D3ED III) in lipidated form using an *Escherichia coli*-based system. The recombinant lipidated dengue-3 envelope protein domain III (LD3ED III) augments the expression levels of IL-12 family cytokines. LD3ED III-immunized mice enhance wide ranges of T cell responses as indicated by IFN-γ, IL-17, IL-21 production. Additionally, LD3ED III-immunized mice increase the frequencies of anti-D3ED III antibody producing cells. The boosted antibody titers cover various IgG isotypes, including IgG1, IgG2a, IgG2b, and IgG3. Importantly, LD3ED III-immunized mice induce neutralizing antibody capacity associated with a reduction of viremia levels after challenges. In contrast, mice that are immunized with D3ED III formulated with aluminum phosphate (D3ED III/Alum) only enhance Th2 responses and boost IgG1 antibody titers. Neither neutralizing antibody responses nor the inhibition of viremia levels after challenge is observed in mice that are immunized with D3ED III/Alum. These results suggest that LD3ED III can induce broad profiles of cellular and humoral immune responses.

1. Introduction

Dengue fever or severe dengue hemorrhagic fever and dengue shock syndrome are caused by the infection of dengue virus, which includes four antigenically different serotypes. Although the true disease burden is not well known, a recent estimation reported that dengue infection reaches 390 million clinical infections annually [1] and results in a serious public health threat in more than 120 countries throughout tropical and subtropical areas [2,3]. It is believed that vaccination is an effective and successful tool to combat pathogens [4]. A licensed dengue vaccine is not currently available due to the complex interaction of four serotypes of dengue virus with the human immune system. Pharmaceutical companies and research laboratories have employed various technologies for dengue vaccine development. Several dengue vaccine candidates are in clinical development [5]. The leading vaccine candidate is Sanofi Pasteur's live chimeric virus vaccine. After three injections of tetravalent live chimeric virus vaccine, the vaccine efficacies were 56.5% [6] and 60.8% [7] in Asia and Latin America phase 3 studies, respectively. Thus, continued efforts are required to develop dengue vaccines.

Subunit vaccines provide an alternative approach for dengue vaccine development. Recombinant protein-based subunit vaccines do not contain live components of pathogens and are considered to be safe. In total, recombinant proteins are poor vaccine immunogens and require appropriate adjuvants to provoke the desired immune responses. When designing a subunit vaccine, the choice of immunogen is determined by the pathogen,

Abbreviations: D3ED III, ecombinant dengue-3 envelope protein domain III; ED III/Alum, D3ED III formulated with aluminum phosphate; FFU, focus-forming unit; FRNT, focus reduction neutralization tests; IMAC, immobilized metal affinity chromatography; LD3ED III, recombinant lipidated dengue-3 envelope protein domain III.

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http://dx.doi.org/10.1016/j.vaccine.2016.01.009

whereas the selection of adjuvant depends on the desired immune response. Dengue envelope protein domain III is a critical region on the viron surface for viral attachment to cellular receptors [8,9]. Several neutralizing epitopes have been identified in dengue envelope protein domain III [10–12]. These results suggest that dengue envelope protein domain III is a suitable target for dengue vaccine development [13]. Several dengue envelope protein domain III subunit vaccine candidates have been evaluated in mice [14–21] and nonhuman primates [22–26]. A subunit vaccine candidate formulated with proper adjuvant is required to induce robust immunity. Unfortunately, formulations of dengue subunit vaccines using adjuvants containing aluminum, the most widely used adjuvants in human vaccines, are unable to induce complete protection against dengue virus infection [17,22,25]. Exploring exogenous adjuvant-independent approaches to enhance immunogenicity may provide a solution for dengue subunit vaccine design.

An efficient way to trigger immune responses is through the presentation of the antigen by antigen-presenting cells and simultaneously providing activation signals [27,28]. To mimic natural infection, recombinant protein is linked to pattern recognition receptor ligands to create immunogen-pattern recognition receptor ligand-conjugated subunit vaccines, ensuring that the same antigen-presenting cell receives immunogens and stimulation signals at the same time. In our previous studies, we developed a novel platform technology to express recombinant lipidated immunogens with intrinsic adjuvant properties [29]. The lipid moiety of recombinant lipidated immunogens provides a danger signal to activate antigen-presenting cells via toll-like receptor 2 [30] and induces an appropriate adaptive immunity in the absence of exogenous adjuvant formulation [19]. The lipidation strategy has been applied to dengue-1, -2, and -4 envelope protein domain III [17,18,20,31], but recombinant lipidated dengue-3 envelope protein domain III (LD3ED III) has not yet been characterized. In the present study, we further dissected the profile of T cell and antibody responses in mice that were immunized with LD3ED III. We confirmed and further extended the main findings of our previous works [17,18,20,31], namely, that recombinant lipidated immunogens can induce strong and persistent immune responses even without formulation with exogenous adjuvants.

2. Materials and methods

2.1. Virus

Dengue-3/H-087 (laboratory-adapted virus) was used for this study and kindly provided by Dr. Yi-Ling Lin of the Institute of Biomedical Sciences, Academia Sinica, Taiwan. Propagation of virus was performed in C6/36 cell, and viral titers were determined by focus-forming assays with BHK-21 cells [21].

2.2. Experimental mice and immunization

Female BALB/c mice were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The mice were maintained at the Laboratory Animal Center of the National Health Research Institutes (NHRI). All of the animal experiments were approved and performed in compliance with the guidelines of the Animal Committee of the NHRI. Groups of mice (6–8 weeks of age) were immunized with D3ED III, D3ED III/Alum, or LD3ED III via subcutaneous injection. Each mouse received a 10 μg/0.2-mL dose. Mice that were immunized with PBS alone (without antigens) served as controls. All of the animals were given 2 injections at a two-week interval with the same regimen. Blood samples were collected by tail bleeding for 0.1–0.2 mL from each mouse at different time points as indicated. The serum samples were prepared and stored at −20 °C until use.

2.3. Preparation of recombinant proteins and the other materials and methods

All the details were in Supplementary data.

3. Results

3.1. Preparation and characterization of dengue-3 envelope protein domain III recombinant immunogens

The dengue-3 envelope protein domain III gene was cloned into the expression vector pET-22b(+) (Novagen, Madison, WI), using Nde I and Xho I sites to produce the pD3DEIII plasmid for the production of recombinant dengue-3 envelope protein domain III (D3E3 III). To produce LD3ED III, the D3ED III gene was cloned into the pET-22b-based plasmid containing a lipid signal peptide using Bam HI and Xho I sites to produce the pLD3EDIII plasmid. As a result, both D3ED III and LD3ED III were contained an additional HHHHHH sequence (HisTag) at their C-terminus and were expressed under the control of the T7 promoter (Fig. 1A).

The D3ED III was purified using an immobilized metal affinity chromatography (IMAC) column (Fig. 1B, lanes 1–4). D3ED III was detected with anti-HisTag antibodies (Fig. 1B, lanes 5–8). The lipidated counterpart of D3ED III, LD3ED III was also purified using an IMAC column (Fig. 1B, lanes 9–12) and was detected with anti-HisTag antibodies (Fig. 1B, lanes 13–16). After removing the lipopolysaccharide (LPS), the residues of LPS in D3ED III and LD3ED III were less than 0.06 EU/mg. To have stable materials for further characterization and validation, we produced and lyophilized a sufficient amount of materials in one batch. Endotoxin-free D3ED III and LD3ED III were comparatively analyzed for their immunogenicity and efficacy in animal models.

Next, the exact mass of the N-terminal fragments of LD3ED III were measured. As shown in Fig. 1C, there were three major peaks with m/z values of 1452, 1466, and 1480 in the spectrum. These peaks have been identified in other lipidated proteins and can be considered as a lipidation signature [18,20,29,31,32]. We confirmed that the peaks of LD3ED III were associated with the lipidated cysteine residue and verified that LD3ED III contains a *N*-acetyl-*S*-diacyl-glyceryl-cysteine at its N-terminus.

3.2. Recombinant lipidated dengue-3 envelope protein domain III upregulates IL-12 family cytokines in bone marrow-derived dendritic cells

Cytokines are important mediators of immune responses. Dendritic cells produce IL-12, IL-23, and IL-27 to modulate T cell differentiation. Members of the IL-12 family are heterodimeric cytokines that consist of an α chain and a β chain. The p40 chain can heterodimerize with p35 or p19 to form IL-12 or IL-23, respectively, whereas Epstein-Barr virus-induced gene 3 (EBI3) can pair with p28 or p35 to form IL-27 or IL-35, respectively [33]. Therefore, IL-12 family cytokines were analyzed after stimulation. The expression levels of p40, p35, p19, p28, and EBI3 mRNA in dendritic cells 4 h after LD3ED III stimulation were higher than PBS stimulated dendritic cells for 7180-, 79-, 1895-, 1563-, and 17-fold, respectively (Fig. 2A). To verify the cytokine expression, heterodimeric form of IL-12, IL-23, and IL-27 levels in the supernatants were evaluated by ELISA. As shown in Fig. 2B, the highest levels of IL-12, IL-23, and IL-27 were induced after LD3ED III stimulation. In contrast, there were no significant IL-12, IL-23, and IL-27 production when dendritic cells were stimulated with 6 EU/mL LPS (equivalent to 10,000 folds of LD3ED III LPS residues) (Supplementary Fig. 1). These results indicate that LD3ED III activates dendritic cells and upregulates the production of IL-12 family cytokines in dendritic

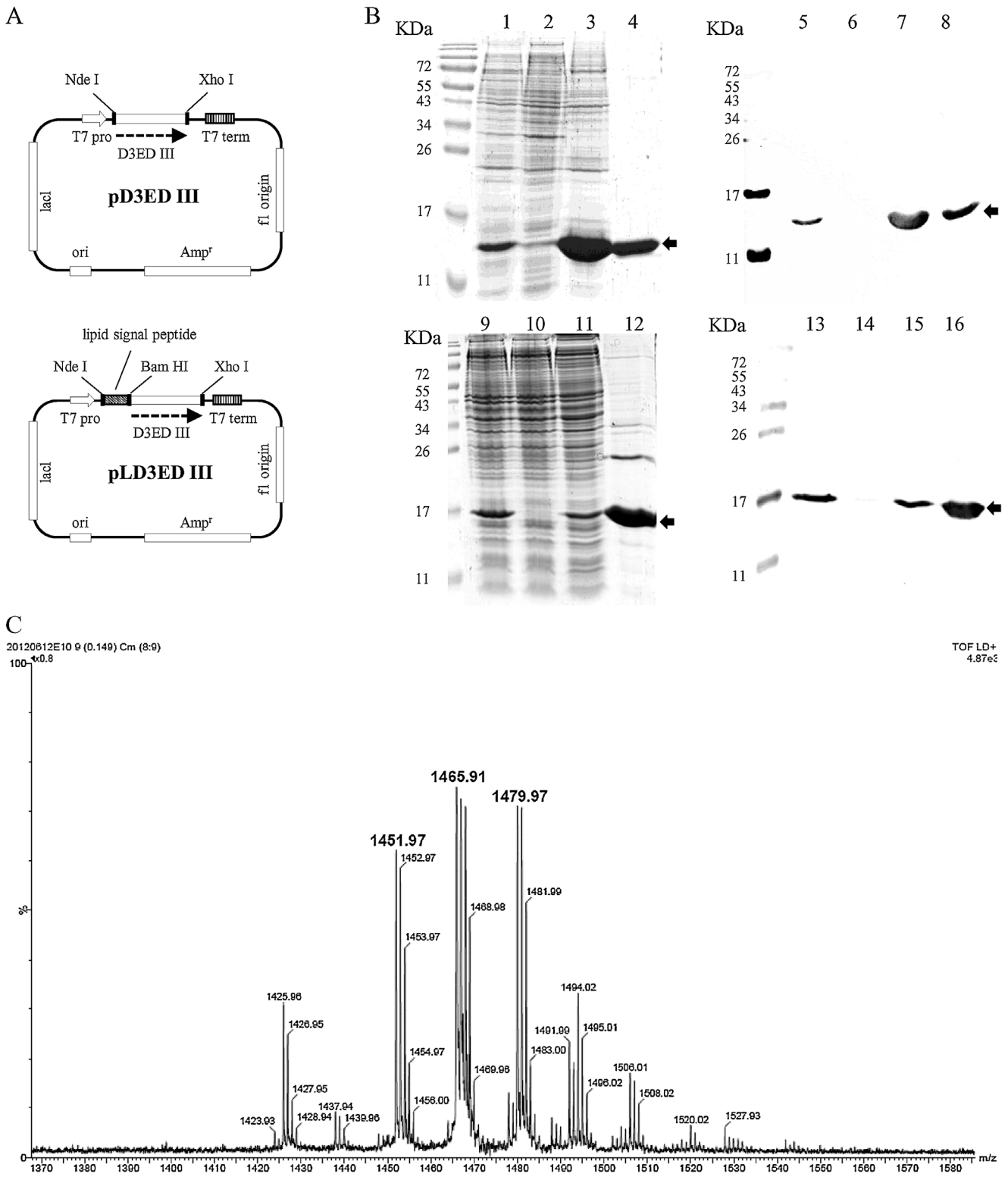

Fig. 1. *Production and identification of D3E3 III and LD3ED III.* (A) Circular map of plasmid pD3ED III and pLD3ED III for the production of D3ED III and LD3ED III, respectively. (B) The purified D3E3 III protein was monitored by 15% reducing SDS-PAGE. Coomassie Blue staining was followed. Anti-HisTag antibodies were used to perform immunoblotting (lanes 1–8). D3E3 III was expressed in *E. coli* strain BL21 (DE3). Lane 1, D3E3 III expression after IPTG induction; lane 2, protein expression in the absence of IPTG induction; lane 3, soluble fraction of D3E3 III; lane 4, purified D3E3 III. Lanes 5–8 demonstrate D3E3 III induction and purification processes by immunoblotting. The samples in these lanes are the same as those in lanes 1–4, respectively. The arrows point the electrophoretic positions of D3E3 III in the gels or blots. The LD3ED III protein purification process was monitored using 15% reducing SDS–PAGE, followed by staining with Coomassie Blue and immunoblotting using anti-HisTag antibodies (lanes 9–16). LD3ED III was expressed in *E. coli* strain C43 (DE3). Lane 9, shows LD3ED III expression after IPTG induction; lane 10, without IPTG induction; lane 11, soluble fraction of LD3ED III; lane 12, purified LD3ED III. Lanes 13–16 represent the result of immunoblotting of LD3ED III induction and purification processes. Samples in these lanes are the same as those in lanes 9–12, respectively. The arrows indicate the electrophoretic positions of LD3ED III in the gels or blots. (C) LD3ED III was digested with trypsin to obtain the N-terminal LD3ED III fragments. The digested sample was analyzed on a Waters® MALDI micro MX™ mass spectrometer. The MALDI-TOF MS spectra revealed the presence of three major peaks with *m/z* values of 1452, 1466, and 1480.

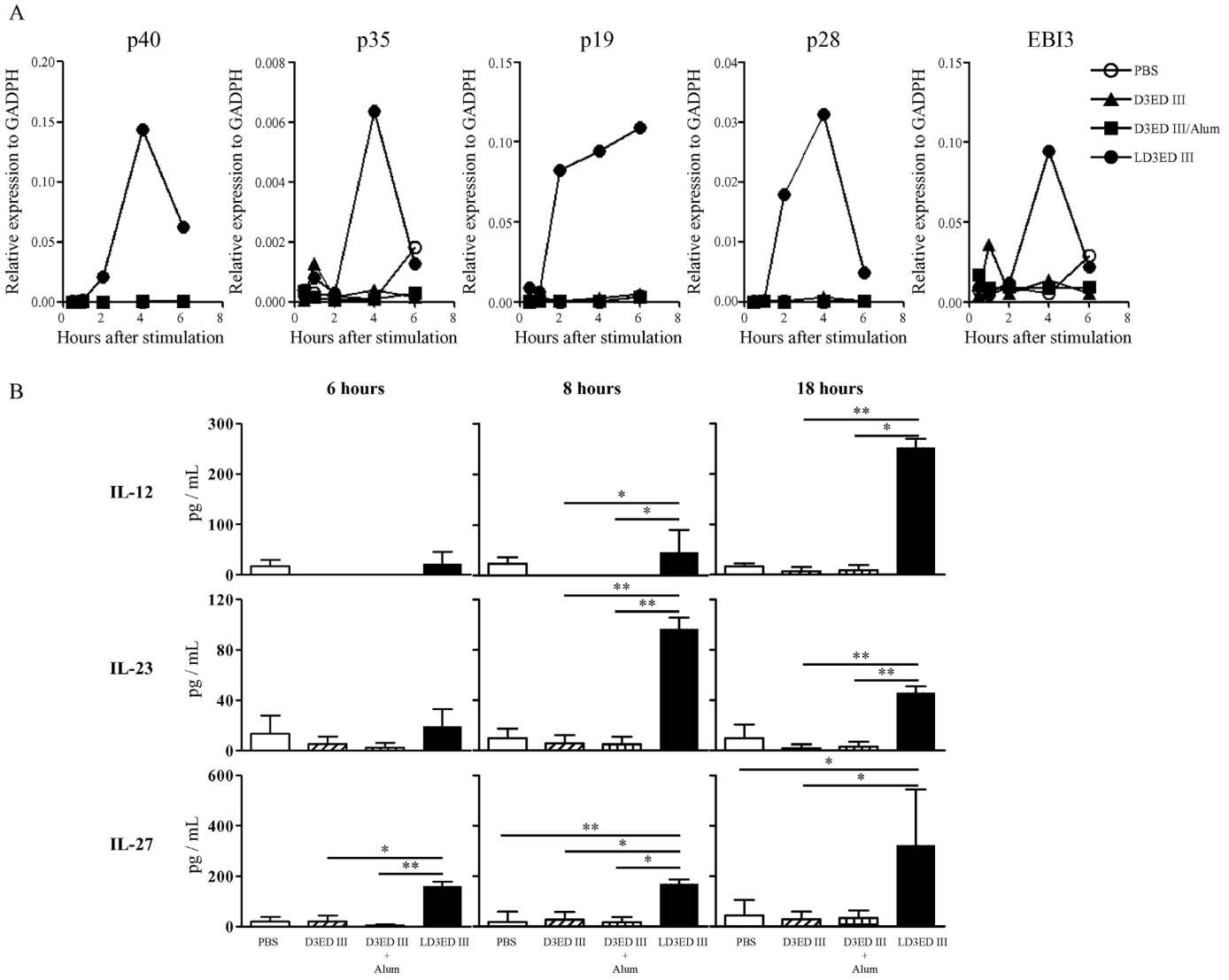

Fig. 2. *The expression profile of IL-12, IL-23 and IL-27 in murine bone marrow-derived dendritic cells.* Mouse dendritic cells from bone marrow progenitor cells were stimulated with 10 μg of D3ED III, D3ED III/Alum, or LD4ED III in PBS. Dendritic cells that were stimulated with PBS alone (without antigens) served as controls. (A) The cells were harvested at 0.5, 1, 2, 4, and 6 h post-stimulation. The RNA was extracted for real-time quantitative PCR analysis. The gene expression was normalized to GAPDH mRNA. The data that are shown represent 2 independent experiments. (B) The supernatants were collected at 6, 8, and 18 h after stimulation. The heterodimeric form of IL-12, IL-23, and IL-27 levels were determined using ELISA kits. The data represent mean ± standard deviation from total 6 wells of 2 independent experiments. The statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparison test. * $p < 0.05$. ** $p < 0.01$.

cells. These effects are not caused by the trivial LPS residues in LD3ED III.

3.3. IFN-γ and IL-17 are induced in mice that are immunized with recombinant lipidated dengue-3 envelope protein domain III

To ascertain whether the expression profiles of cytokine genes on bone marrow-derived dendritic cells associate *in vitro* with the immune responses *in vivo*, we immunized mice with various formulations and evaluated the immune responses after immunization. Groups of BALB/c mice were immunized with D3ED III, LD3ED III or D3ED III formulated with aluminum phosphate (D3ED III/Alum) two times at a two-week interval. The animals that were immunized with PBS alone served as negative controls. One week after the last immunization, the splenocytes were examined for cytokine secretion. Fig. 3 shows that supernatants that were obtained from all of the splenocytes produced little levels of IFN-γ, IL-5, and IL-17 without stimulation (medium alone) or stimulated with bovine serum albumin (BSA) for all the time points tested. Remarkably, the supernatants that were obtained from D3ED III-stimulated splenocytes induced different cytokine profiles. The levels of IFN-γ and IL-17 were higher in LD3ED III-immunized mice than in D3ED III-, D3ED III/Alum-, or PBS-immunized mice. Interestingly, the levels of IL-5 were higher in the mice that were immunized with D3ED III/Alum than in mice that were immunized with PBS, D3ED III or LD3ED III. These results suggest that LD3ED III elicits different cellular immune response profiles compared to D3ED III/Alum.

3.4. Enhanced humoral immunities are induced in mice that are immunized with recombinant lipidated dengue-3 envelope protein domain III

IL-21 plays an important role in regulating B cell differentiation, plasma cell generation, and antibody production [34,35]. IL-21-producing cells in spleens were examined by ELISPOT. As shown in Fig. 4A, the cultured cells without stimulation (medium alone) or stimulated with BSA did not induced IL-21 secretion.

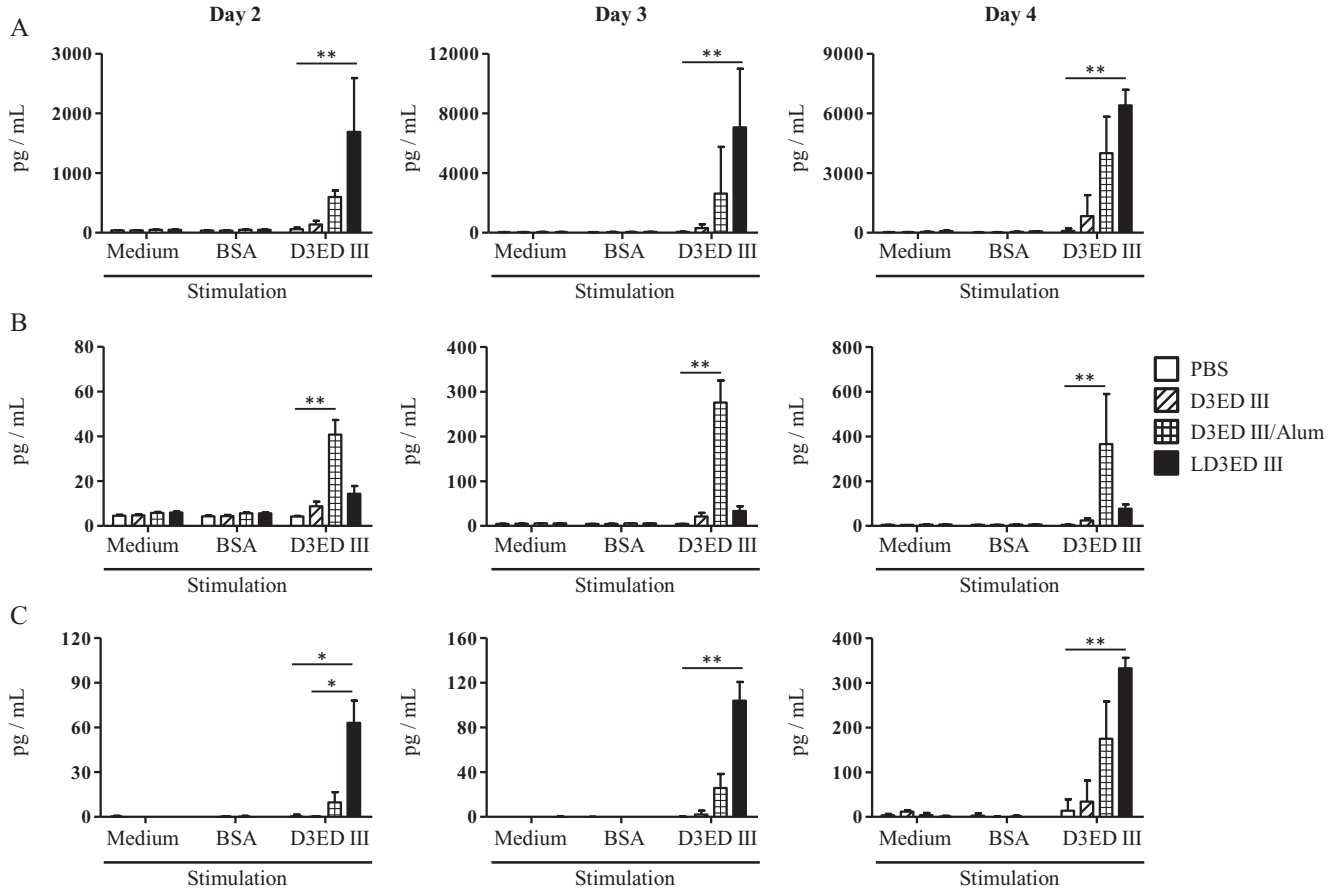

Fig. 3. *The cytokine production profile in D3ED III- (with or without aluminum phosphate) and LD3ED III-immunized mice.* Groups of BALB/c mice were immunized subcutaneously twice with 10 μg of D3ED III, D3ED III/Alum, or LD4ED III in PBS at a 2-week interval. Mice that were immunized with PBS alone (without antigens) served as controls. Splenocytes were harvested one week after the second immunization. The cells were cultured without stimulation or stimulated with BSA or D3ED III for 2, 3, and 4 days. The supernatants were collected and used to evaluate the levels of (A) IFN-γ, (B) IL-5, and (C) IL-17 by ELISA. Data represent mean ± standard deviation from 4 mice. The statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparison test. * $p < 0.05$. ** $p < 0.01$.

Interestingly, IL-21 secretion was induced in response to D3ED III stimulation in mice that were immunized with LD3ED III but not mice that were immunized with PBS, D3ED III, or D3ED III/Alum. The incidence of anti-D3ED III antibody-producing cells following immunization with LD3ED III was assessed by ELISPOT. An increase in anti-D3ED III antibody-secreting cells in bone marrows compared to anti-D3ED III antibody-secreting cells in mice that were immunized with PBS, D3ED III, or D3ED III/Alum was obtained and was statistically significant (Fig. 4B, $p < 0.0001$, Kruskal–Wallis test). The incidence of anti-D3ED III antibody-producing cells in the spleens presented a similar profile (Fig. 4C). Overall, we concluded that LD3ED III-immunized mice induce IL-21 secretion and enhance the incidence of B cells to produce anti-D3ED III antibodies.

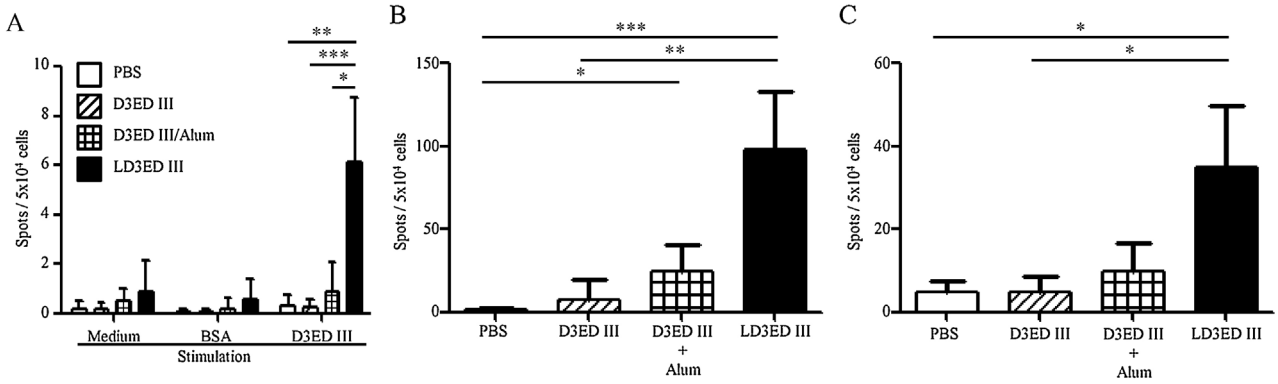

Fig. 4. *Identification of IL-21-producing cells and D3ED III-specific B cells in D3ED III-(with or without aluminum phosphate) and LD3ED III-immunized mice.* Groups of BALB/c mice were immunized subcutaneously twice with 10 μg of D3ED III, D3ED III/Alum, or LD4ED III in PBS at a 2-week interval. Mice that were immunized with PBS alone (without antigens) served as controls. Splenocytes and bone marrow cells were harvested one week after the second immunization. (A) IL-21-producing cells in spleens, (B) D3ED III-specific antibody-secreting cells in bone marrows and (C) spleens were evaluated by ELISPOT. Data represent mean ± standard deviation from total 7–8 mice of 2 experiments. The statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparison test. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

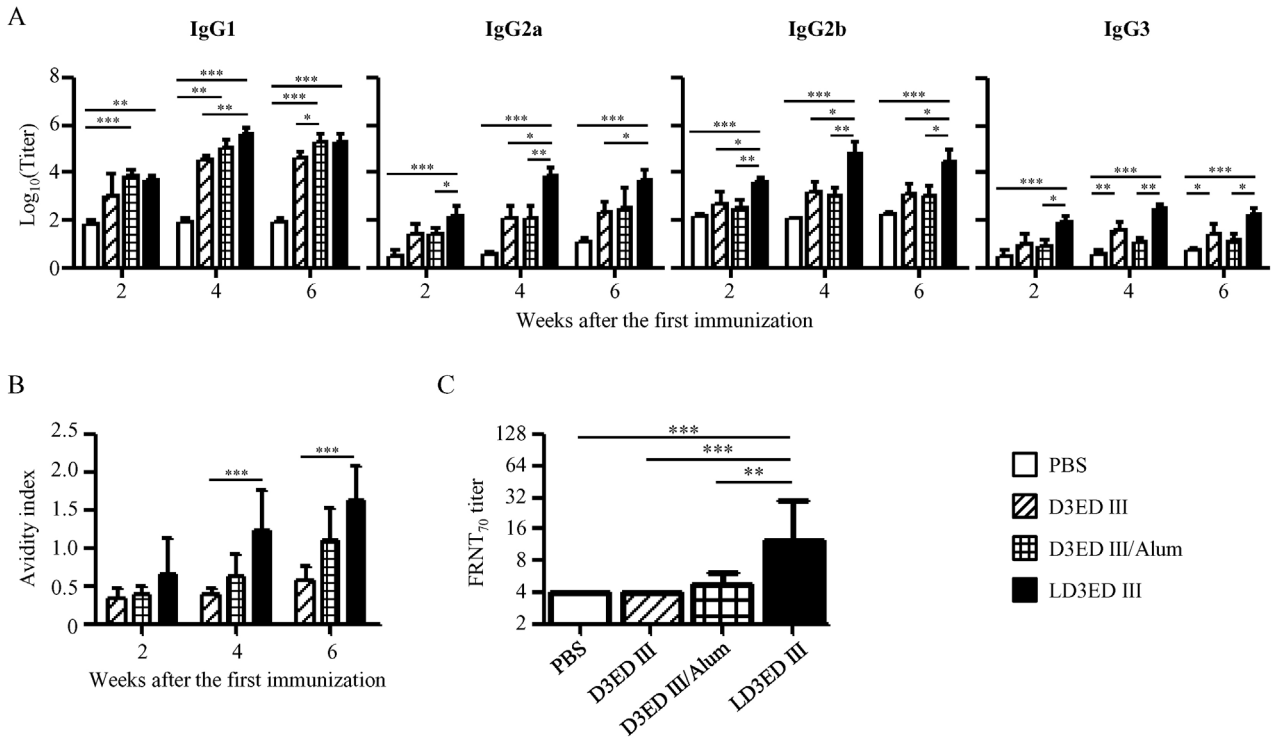

Fig. 5. *Analysis of antibody responses in D3ED III- (with or without aluminum phosphate) and LD3ED III-immunized mice.* Groups of BALB/c mice were immunized subcutaneously twice with 10 μg of D3ED III, D3ED III/Alum, or LD4ED III in PBS at a 2-week interval. Mice that were immunized with PBS alone (without antigens) served as controls. The sera were collected from mice at the indicated time points after the first immunization. (A) Subclasses of anti-D3ED III IgG antibodies and (B) antibody avidity profiles were examined by ELISA. (C) The capacity of dengue-3 virus-neutralizing antibody was determined by FRNT 8 weeks after the first immunization. The neutralizing antibody titer was calculated as the highest dilution that resulted in a 70% reduction in FFU compared to that of the control samples containing the virus alone. The antibody titers were logarithmically transformed before statistical analyses. Data represent mean ± standard deviation from total 8–9 mice of 2 experiments. The statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparison test. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

The anti-D3ED III IgG subclass profiles of serum samples that were collected from different groups at weeks 2, 4, and 6 were examined. Of the 4 groups of mice, LD3ED III-immunized mice generated the highest IgG antibody responses for all the subclasses that we examined. Notably, the mice that were immunized with D3ED III/Alum increased the anti-D3ED III IgG1 titers but not those of IgG2a, IgG2b, and IgG3 compared to mice that were immunized with D3ED III without adjuvant (Fig. 5A). We next evaluated the antibody avidity profiles of the serum samples. There was no significant difference in the avidity index among groups of mice 2 weeks after the first immunization. After the second immunization, the avidity index of sera that were obtained from the mice that were immunized with LD3ED III quickly increased and was significantly higher than that of the sera that were obtained from the mice that were immunized with D3ED III ($p < 0.001$, Kruskal-Wallis test with Dunn's multiple comparison test). However, the avidity index of the sera that were obtained from the mice that were immunized with D3ED III/Alum was not significantly higher than that of the sera that were obtained from mice that were immunized with D3ED III 2 and 4 weeks after booster immunization (Fig. 5B). Altogether, these results suggest that LD3ED III can increase the responses of various IgG subclasses and enhance antibody affinity.

3.5. *Lipidated dengue-3 envelope protein domain III induces neutralizing antibodies and reduces the viremia levels after challenge*

To determine whether mice that are immunized with LD3ED III could elicit immunity to blocked viral infection and clear viral load in the blood, neutralizing antibody titers induced by vaccination and viremia levels after challenge were examined. Serum samples were collected at weeks 8 after the first immunization. The neutralizing capacity of antibodies was determined by focus reduction neutralization tests. As shown in Fig. 5C, the mice that were immunized with D3ED III or D3ED III/Alum could not stimulate significant neutralizing antibody responses ($FRNT_{70} < 8$), similar to mice that were immunized with PBS. In contrast, the mice that were immunized with LD3ED III elicited low but moderate neutralizing antibody responses (average $FRNT_{70} = 12.7$) ($p < 0.001$, Kruskal–Wallis test). Next, all of the immunized mice were challenged with dengue-3-infected K562 cells 20 weeks after the first immunization. The viremia levels of mice that were immunized with D3ED III or D3ED III/Alum were equivalent to those of mice that were immunized with PBS at all of the time points that we examined. Although the viral loads in the blood of LD3ED III-immunized mice were comparable to the other vaccinated mice 8 h after challenge, the viremia levels of LD3ED III-immunized mice quickly decreased and were significantly lower than those the other vaccinated mice 22 ($p < 0.01$, Kruskal–Wallis test) and 32 ($p < 0.05$, Kruskal–Wallis test) hours after challenge (Fig. 6). Collectively, our results suggest that LD3ED III induces neutralizing antibodies and reduces the viremia levels after challenge in mice.

4. Discussion

The development of an effective vaccine that induces a broad and durable adaptive immunity against pathogens is still a serious challenge. The present study, using LD3ED III, has demonstrated that immunogens that are expressed in lipidated form to activate antigen-presenting cells provide an attractive strategy for modulating immune responses and have the potential to enhance the efficacy of vaccines.

The initial adaptive immune responses are triggered by professional antigen-presenting cells. Dendritic cells are the most potent antigen-presenting cells to activate naïve T cells [36]. Upon activation, dendritic cells can produce different cytokines depending on the stimuli to modulate immune responses. IL-12, IL-23, and IL-27, the members of the IL-12 cytokine family that are produced by dendritic cells, are particularly influential in determining the fate of T cells [33]. We demonstrate that LD3ED III induces the upregulation of all of the components of IL-12 cytokine family mRNA (Fig. 2A). Importantly, IL-12, IL-23, and IL-27 levels in the supernatants are augmented under the stimulus of LD3ED III (Fig. 2B). IL-12 and IL-23 are key cytokines in facilitating Th1 differentiation [37] and supporting Th17 expansion [38], respectively. In agreement with these findings, mice that are immunized with LD3ED III elicit IFN-γ and IL-17 production (Fig. 3), which are the signature cytokines of the Th1 and Th17 response, respectively. Many studies have reported that IL-27 acts to constrain inflammation in most cases. However, IL-27 plays a proinflammatory role in some circumstances [39]. IL-27 facilitates the development of follicular helper T cells via the induction of IL-21 [40]. IL-21 can also act as an autocrine growth factor for follicular helper T cells [41,42]. In the present study, IL-21-producing cells were induced in mice that were immunized with LD3ED III but not D3ED III or D3ED III/Alum (Fig. 4A). Altogether, these findings suggest that recombinant lipidated immunogen stimulates antigen-presenting cells to upregulate IL-12, IL-23, and IL-27 production. Under these conditions, the development of Th1, Th17, and follicular helper T cells is efficiently promoted.

The follicular helper T cell-derived IL-21 provides crucial signals to modulate the development and function of B cells [43,44]. The IL-27-IL-21 axis is important for the germinal center function and development of T cell-dependent antibody responses [40]. Germinal centers are the sites of B cell clonal expansion, somatic hypermutation, and affinity maturation, the combination of which results in the production of high-affinity antibodies [45]. The affinity of antibodies in mice that were immunized with LD3ED III was quickly increased and higher than that in mice that were immunized with D3ED III formulated with or without aluminum phosphate (Fig. 5B). These results indicate that LD3ED III-immunized mice build a favorable environment for B cell affinity-based selection. Antibodies are secreted by plasma B cells. The long-lived plasma B cells migrate to the bone marrow, which is responsible for long-term humoral immunity [46]. Notable frequencies of D3ED III-specific antibody-secreting cells are present in the bone marrow and spleen of LD3ED III-immunized mice (Fig. 4B and C). In addition, a comprehensive enhancement of antibody responses is induced in mice that are immunized with LD3ED III. The elevation of antibody titers is not limited to IgG1 but also other IgG isotypes, including IgG2a, IgG2b, and IgG3 (Fig. 5A). High IgG1 titers for the dengue envelope protein domain III have been shown in mice immunized with subunit vaccines. Interestingly, low titers of IgG3 against dengue envelope protein domain III were only detected in mice after immunization with dengue virus [16,47]. These results suggest that LD3ED III elicits a much more diverse IgG subclass response which is similar to dengue virus. In contrast, mice that are immunized with D3ED III/Alum induce the production of IL-5, a Th2 cytokine (Fig. 3), and only enhance IgG1 antibody responses (Fig. 5A). Associated with these findings with our cytokine results in dendritic cells and T cells, mice that are immunized with LD3ED III should promote the efficient production of anti-D3ED III-specific antibodies. We conclude that mice that are immunized with LD3ED III not only increase the intensity of antibody responses (antibody titers) but also broaden the profile of antibody responses (IgG isotypes) and enhance the antibody affinity.

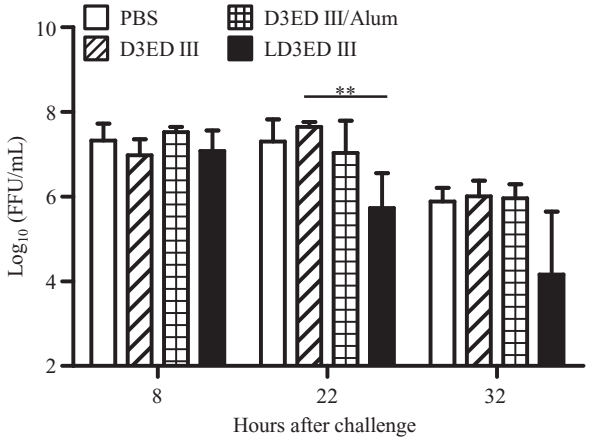

Fig. 6. *Inhibition of viremia levels in LD3ED III-immunized mice.* BALB/c mice were immunized subcutaneously twice with 10 μg of D3ED III, D3ED III/Alum, or LD3ED III in PBS at a 2-week interval (5 mice for each group). Mice that were immunized with PBS alone (without antigens) served as controls. Twenty weeks after the first immunization, the mice were intraperitoneally challenged with dengue-3-infected K562 cells. The mice were bled after challenge at the indicated time points ($n = 5$ for each time point). The plasma viral titers were determined by focus-forming assays using BHK-21 cells. The viremia levels were logarithmically transformed before statistical analyses. Data represent the mean ± standard deviation. The statistical significance was determined by Kruskal–Wallis test with Dunn's multiple comparison test. ** $p < 0.01$.

Mice immunized with LD3ED III induced low neutralizing antibody titers (average $FRNT_{70} = 12.7$) at 8 weeks after the first immunization (Fig. 5C). However, high levels of anti-D3ED III IgG antibodies were elicited (Fig. 5A). This phenomenon is similar to mice immunized with recombinant lipidated dengue-4 envelope protein domain III (LD4ED III) [31]. It is possible that neutralizing epitopes are not dominant in D3ED III and D4ED III. In general, laboratory strains of immunocompetent mice are not susceptible to clinical diseases. Dengue viral infection may produce viremia in some immunodeficiency mice. Because immunodeficiency mice lack for a normal immune system, they are not suitable for vaccine evaluation. Recently, Yamanaka and Konishi developed a simple model for dengue vaccine evaluation using dengue-infected cells to challenge immunocompetent mice [48]. Thus, we adopted this dengue viral challenge model. Results in the challenge experiments indicate that mice immunized with LD3ED III have better capability of clearance of viral load in the blood than mice immunized with PBS, D3ED III, or D3ED III/Alum (Fig. 6).

Effective vaccination should induce protective immunity. Although D3ED III/Alum can enhance the Th2 response and IgG1 titers, no significant neutralizing capacity (Fig. 5C) or inhibition of viremia were generated (Fig. 6). Remarkably, LD3ED III in the absence of exogenous adjuvant can induce neutralizing antibody responses and reduce viremia levels after challenge. Collectively, LD3ED III alone is able to nourish potent immunity. Subunit vaccines may have some advantages over other vaccine approaches. In general, the major weakness of subunit vaccine is low immunogenicity. An adjuvant is necessary to enhance immune responses. The likely problem does not seem insurmountable, and many solutions could emerge from a detailed study of the lipidated immunogens. Our results suggest that the use of recombinant lipidated immunogens to enhance immunity and improve subunit vaccines therefore has sufficient promise to justify further detailed clinical studies.

Conflict of interest statement

HWC, CHL, SJL, and PC are named on patents relating to the lipidated vaccine against dengue virus infection.

Acknowledgement

This study was financially supported by grants VC-101-PP-26, IV-102-PP-26 and IV-103-PP-16 from the National Health Research Institutes.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.01.009.

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